Probiotic supplementation influences the diversity of the intestinal microbiota during early stages of farmed Senegalese sole (Solea senegalensis, Kaup 1858)

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Probiotic supplementation influences the diversity of the intestinal microbiota during early stages of farmed Senegalese sole (*Solea senegalensis*, Kaup 1858)

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Abstract

Ingestion of bacteria at early stages results in establishment of a primary intestinal microbiota which likely undergoes several stages along fish life. The role of this intestinal microbiota regulating body functions is crucial for larval development. Probiotics have been proved to modulate this microbiota and exert antagonistic effects against fish pathogens. In the present study we aimed to determine ~~microbial~~ bacterial diversity along different developmental stages of farmed Senegalese sole (*Solea senegalensis*) after feeding probiotic (*Shewanella putrefaciens* Pdp11) supplemented diet for a short period (10-30 days after hatching, DAH).

Intestinal lumen contents of sole larvae fed control and probiotic diets were collected at 23, 56, 87 and 119 DAH and DNA was amplified using 16S rDNA bacterial domain-specific primers. Amplicons obtained were separated by Denaturing Gradient Gel Electrophoresis (DGGE), cloned and ~~identified~~ resulting sequences compared to sequences in GenBank. Results suggest that *S. putrefaciens* Pdp11 ~~stabilizes~~ induces a modulation of the dominant bacterial taxa of the intestinal microbiota from 23 DAH. DGGE patterns of larvae fed the probiotic diet showed a core of bands related to *Lactobacillus helveticus, Pseudomonas acephalitica, Vibrio parahaemolyticus* and *Shewanella* genus, together with increased *Vibrio* genus presence. In addition, decreased number of clones related to *Photobacterium damselae* subsp *piscicida* at 23 and 56 DAH was observed in probiotic fed larvae. A band corresponding to *S. putrefaciens* Pdp11 was sequenced as predominant from 23 to 119 DAH samples, confirming the colonization by the probiotics. Microbiota modulation obtained via probiotics addition emerges as an effective tool to improve *S. senegalensis* larviculture.

Keywords: *Solea senegalensis*, probiotics, larviculture, intestinal microbiota

Introduction

Aquaculture has been the fastest growing food-production sector in the world over the last 30 years. During this time farmers have been mainly focused in easily produced remunerative species. Despite recent significant advances on weaning techniques and larvae feeding (Conceicao et al. 2007; Engrola et al. 2009), the production of high quality sole juveniles is still a bottleneck (Dámaso-Rodrigues et al. 2010). Fish larvae are exposed to microbiota-associated disorders because they start feeding when the digestive tract is not fully developed (Rodríguez et al. 2009). The fFish intestinal tract is an ecosystem containing microbial ~~communities~~ assemblages involved in very important functions such as the integrity of the epithelial surface, stimulation of the epithelial proliferation, nutrition and digestion via the production of a range of vitamins and enzymes (Ramirez and Dixon 2003; Ringø et al. 2004; 2007). The intestinal microbiota ~~al community~~ also plays a crucial role on the host immunity through the immunostimulation and development of gut-associated lymphoid tissues (Picchietti et al. 2007; Rombout et al. 2011), with important functions in the outbreak of diseases. As the gastrointestinal tract serves as a route for entry of pathogenic microorganisms (Chen et al. 2008; Ringø et al. 2004), disruptions of its ecologic balance, increased permeability and deficiencies in immune defences may lead to bacterial translocation of pathogenic microorganisms (Ringø et al. 2007; Sekirov and Finley, 2009).

In this context, the application of probiotics to fish larviculture has been extensively established based on health parameters and growth performance (Avella et al. 2010; Carnevali et al. 2006; Gatesoupe 2008; Tinh et al. 2008), although the modes of action involved are largely unknown (Merrifield et al. 2010; Askarian et al. 2011). Microbial modulation of the autochthonous intestinal microbiota may be considered as one of these modes of action (Fergusson et al. 2010; Merrifield et al. 2010).

Shewanella putrefaciens Pdp11 has been proposed as a species exhibiting remarkable probiotic characteristics, including antagonism against some of the most important pathogens for Senegalese sole *Solea senegalensis* (Chabrillón et al. 2005a,b) and increasing fish resistance against *Photobacterium damselae* subsp *piscicida* (Díaz-Rosales et al. 2009; García de la Banda et al. 2010; 2012). Moreover, *S. putrefaciens* Pdp11 was reported to protect intestinal epithelium integrity of sole juveniles (García de la Banda et al. 2010; Sáenz de Rodrigáñez et al. 2009), enhancing growth and biochemical composition both at ongrowing (García de la Banda et al. 2012) and larval rearing (Lobo et al. 2014). However, a general understanding of the microbiotaal ~~communities~~ associated with *S. senegalensis* in the first stages of development is necessary to improve rearing performances and adequate use of probiotics.

In this study we describe the variability and succession of the predominant bacterial taxa of the intestinal microbiota ~~during *S. senegalensis*~~ in the first stages of *S. senegalensis* development~~, a marketable candidate for marine intensive farming~~. In addition, administration of a short probiotic pulse during metamorphosis (10-30 DAH) was conducted to evaluate effects on intestinal microbiota of farmed sole larvae and fry.

Material and Methods

Microorganisms

Shewanella putrefaciens Pdp11 cells were stored at -80ºC in tryptone soya broth (Oxoid Ltd., Basingstoke, UK) supplemented with 15 gl^{-1} NaCl (TSBs) and 15% glycerol for long-term storage. For the experiments, bacterial strains were grown in tubes containing 5 ml (TSBs) for 18 h at 22 ºC, with continuous shaking. Appropriate dilutions of the cultures were spread onto tryptone soya agar (Difco™, Sparks, USA) plates supplemented with 1.5 % NaCl (TSAs) and confirmed as Pdp11 according to Diaz-Rosales et al. (2009). Aliquots of 0.1 ml of the culture were spread onto TSAs plates and incubated for 24h at 22ºC. Bacterial suspensions were prepared by scraping cells from the plates and washing them in sterile phosphate-buffered saline (PBS, pH 7.4), and finally added to *Artemia* (2~~.5x10^{7} CFU~~00-300 metanauplii ml^{-1}) at least three hours prior to larval feeding at $2.5x10^{7}$CFU ml^{-1}~~.~~ (final concentration). ~~The number of bacterial cells~~Bacterial concentration ~~ml^{-1}~~ was ~~measured by using a Hach DR/2500 Laboratory Spectrophotometer (Loveland, Colorado, USA).~~ determined by count on TSAs plates. The bacterial dose has been previously reported as suitable by Lobo et al. (2014) and is in the range of other probiotics used in larviculture (Dias et al., 2011; Hernández-Martínez et al., 2009). No probiotic cells were supplied to the *Artemia* incubator used as control. After an incubation period of 3-9 h the concentration of total bacteria was 10^{4} CFU *Artemia* metanauplii^{-1} where *S. putrefaciens* Pdp11 cells accounted at least for 50%. These values are similar to those reported by other authors (Villamil et al., 2003; Carnevali et al., 2004). No mortalities of *Artemia* metanauplii were registered during the incubation period. *Artemia* from both incubators (45 l) were maintained at 23ºC and rinsed with 1 µm filtered seawater for five minutes prior to their supply to rearing tanks.

Rearing conditions

Embryos were obtained from natural spawning of captive *S. senegalensis* broodstock kept at the Spanish Institute of Oceanography (Santander, Spain). Embryos were incubated at 19.0 ± 0.5 ºC in 70 l cylinder-conical incubating tanks with gentle aeration and 0.5 l min^{-1} continuous seawater flow. Newly hatched larvae (40 individuals l^{-1}) were distributed into 250 L circular polyester tanks, with constant aeration and seawater renewal. Temperature was 18.6 ± 1.1ºC and salinity was 35.4 gl^{-1} throughout the trial. Illumination (1000 lux on surface water) was continuous until 10 DAH and a 12:12 L:D cycle was established until 21 DAH, whereas 0:24 L:D was used thereafter. Continuous water inflow was maintained to provide suitable oxygen and nitrite seawater levels for larval and postlarval culture (Lund et al. 2007; Parra and Yúfera 1999).

Feeding regime was based on Cañavate and Fernández-Díaz (1999). *Isochrysis galbana* enriched rotifers were added to the tanks twice a day, from 3 to 9 DAH, to maintain a rotifer density of 20 individuals ml^{-1}. Microalgae (*Nannochloropsis gaditana,* 3×10^{5} cells ml^{-1} and *I. galbana*, 7×10^{4} cells ml^{-1}) were also supplied during this period to ensure good rotifer quality. Co-feeding regimen consisting in *Artemia* and commercial pellet Gemma Micro Diamond (57% crude protein and 15% total lipids,

Skretting, Burgos, Spain) was applied from 10 to 57 DAH. *Artemia* metanauplii (EG strain INVE Aquaculture, Ghent, Belgium) were previously enriched with Origreen (43% fresh protein and 30% total lipids, Skretting, Burgos, Spain) for eighteen hours, and then supplied to the tanks four times a day, whereas dry feed was supplied eight times a day. Weaning started at 58 DAH (Gemma Wean Diamond, crude protein 60% and total lipids 15%, Skretting, Burgos, Spain). Larvae were fed exclusively dry feed (Gemma Diamond) from 87 DAH. The amount of inert feed was gradually increased from 58 DAH (39 g m^{-2}, 69.2 % total feed) to 87 DAH (117 g m^{-2}), while *Artemia* doses were progressively reduced from 14 metanauplii ml^{-1}. At the end of the trial, sole fries were fed 11.3% total tank biomass. Two live feeding regimes were compared: Pdp11 and Control. Pdp11 group consisted of *S. putrefaciens* Pdp11 bacterial strain bioencapsulated in Origreen enriched *Artemia*. The treatment was given three times a day (from 10 to 30 DAH), whereas no bacteria were administered to the Control group. From 30 DAH and until the end of weaning all larvae were fed non supplemented *Artemia*. Each dietary treatment was evaluated by triplicate.

Digestive Microbiota Analysis

Six larvae from each batch were collected on days 23 (end of methamorphosis), 56 (beginning of weaning), 87 (end of weaning) and 119 DAH and digestive microbiota was studied. The whole intestines were aseptically removed and stored at -20ºC until further analysis. The intestinal contents were homogenized in 1 ml PBS (pH 7.2), and 1ml aliquot was centrifuged at 1000×g for 5min. Total DNA was extracted from samples according to Martínez et al. (1998), with some modifications as described by Tapia-Paniagua et al. (2010). Agarose gel (1.5% [wt/vol]) electrophoresis in the presence of ethidium bromide was used to visually check for DNA quality and yield. DNA from an axenic culture of *S. putrefaciens* Pdp11 in TSBs was used as positive control for the presence of the probiotics in intestinal samples.

PCR Denaturing Gradient Gel Electrophoresis Analysis

DNA was amplified using the 16S rDNA bacterial domain-specific primers 968-GC-F (5'GA-ACGCGAAGAACCTTAC-3') and 1401-R (5'CGGTGTGTACAAGACCC-3'). Primer 968-CG-F carries a 35 bp GC clamp. Both primers amplify V6-V8 regions of 16S rDNA, and yield 470-bp length amplicons. PCR mixtures and conditions to perform PCR were those previously described by Tapia-Paniagua et al. (2010). The amplicons obtained were separated by ~~Denaturing Gradient Gel Electrophoresis (~~DGGE~~)~~ according to Muyzer et al. (1993) specifications using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA). The gels were subsequently stained with $AgNO_3$ (Sanguinetti et al. 1994). A DGGE analysis of all samples was performed twice.

Structural diversity of the microbial community was determined based on the DGGE-patterns, which were analyzed using FPQuest Software version 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). A matrix of similarities using Bray-Curtis index was calculated for densitometric curves and a similarity matrix was obtained. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA). In order to be able to compare intestinal microbial communities, several parameters were evaluated: (1) Species

richness (R), based on the total number of bands; (2) Shannon diversity index (H'), according to the function: H′=−Σ Pi log Pi, where *Pi* is defined as (ni/N), *ni* is the peak surface of each band, and *N* is the sum of the peak surfaces of all bands; and (3) Range-weighted richness (Rr) (Marzorati et al. 2008), calculated as the total number of bands multiplied by the percentage of denaturing gradient needed to describe the total diversity of the sample analysed, following the formula: $Rr = (N^2 \times D_g)$, where *N* represents the total number of bands in the pattern, and D_g the denaturing gradient comprised between the first and the last band of the pattern.

Cloning of PCR amplified products

DNA extracted from larvae sampled at each stage was pooled before performing the cloning process. Bacterial 16S rDNA was amplified by PCR using forward primer S-D-Bact-0008-aS-20 (5' AGA GTT TGA TCC TGG CTC AG 3') (Hicks et al. 1992) and reverse primer S-*-Univ-1492-b-A-21 (59ACG GCT ACC TTG TTA CGA CTT 3') (Kane et al. 1993), with Taq DNA polymerase kit from Life Technologies (Gaithersburg, MD, USA), 20mM Tris–HCl (pH 8.5), 50mM KCl, 3mM $MgCl_2$). Amplification was carried out as described by Gray and Herwig, (1996). Reactions were performed in tubes containing 100ng total DNA, 1.25U Taq DNA polymerase, 2.5mM $MgCl_2$ buffer, 200mM each deoxyribonucleotide triphosphate and 10µM each primer in 50µl final volume. Initial DNA denaturation and enzyme activation steps were performed at 94°C for 10min in a Eppendorf thermocycler, followed by 30 cycles denaturation at 92°C for 1min, annealing at 48°C for 1min, and elongation at 72°C for 1min 30s, and final elongation at 72°C for 5min. PCR products were purified and concentrated with High Pure Spin Kit PCR purification kit (Roche) according to manufacturer instructions. Purified PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). Ligation was performed overnight at 4ºC followed by transformation into competent *E. coli* JM109. One hundred ampicillin-resistant transformant colonies from each diet and time sampled were transferred with a sterile toothpick to 100 µl TE buffer and boiled for 10 min at 95ºC. Then, PCR was performed with pGEMT- specific primers T7 (5'-AAT ACG ACT CAC TAT AGG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3'). Plasmids containing an insert were used to amplify 16S rDNA V6-V8 region. Amplicons were compared with the bands of DGGE patterns and used for sequence analysis (Macrogen Korea sequencing).

Statistical analysis

Bray-Curtis similarity index was calculated based on DGGE densitometric curves and used for cluster analysis (UPGMA method) and Non Metric Multidimensional (NMDS) plots. Analysis of similarities (ANOSIM) to test for significant differences between control and treatment groups was carried out using PAST software. Contribution of OTUs identified from cloning experiments to similarity percentages (SIMPER) between control and treatment groups was determined by using PAST software. Significance of differences in Shannon index, species richness and range-weighted richness (Rr) was determined after performing analysis of variance (ANOVA) with XLSTAT 2013 software (Addinsoft, Spain).

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Results

Intestinal bacterial communities from six individuals per treatment and development stage have been analyzed using PCR-DGGE to obtain an overview of bacterial diversity, and 16S rDNA clone libraries have been performed to reveal higher resolution of microbiota composition in both control and probiotic treated larvae.

Cluster analysis was carried out using UPGMA methods based on Bray-Curtis similarity of densitometric 16S rDNA PCR-DGGE curves. Band patterns from 23 DAH (end of metamorphosis) larval samples resulted in two clusters grouping animals fed probiotic and control diet (54% similarity) (Fig. 1A). Each cluster consisted of 5 out of 6 individuals of each treatment; however, no statistically significant differences ($p<0.005$) were visualized when NMDS analysis and ANOSIM were performed comparing intestinal bacterial communities of probiotic and control samples (Fig. 2).

On the contrary, 56 DAH (start of weaning) and 87 DAH (end of weaning) larval PCR-DGGE patterns clustered in two separated major groups, one including control larvae (80% similarity) and the other comprising *S. putrefaciens* Pdp11 fed larvae (84% and 88% similarity, respectively) (Fig 1B and 1C). These two clusters were not observed in 119 DAH animals, when intra-treatment similarity decreased and *S. putrefaciens* Pdp11 and control specimens grouped together again (Fig. 1D). In this case, significant differences ($p<0.005$) were visualized by the NMDS plot and confirmed using ANOSIM for 56 and 87 DAH samples whilst populations of probiotic and control groups overlapped in 119 DAH samples (Fig. 2).

Average numbers of operational taxonomic units (OTUs) or species richness as determined by DGGE bands ranged from 23.0 ± 5.0 to 31.3 ± 3.0 bands in all intestinal samples. No significant differences were observed in species richness regarding the feeding treatment (Table 1). However, significantly lower diversity expressed as Shannon index was observed in the intestines from 56 DAH (2.1 ± 0.2) and 87 DAH (2.1 ± 0.1) larvae fed the probiotic diet compared to their control groups (2.6 ± 0.1 and 2.6 ± 0.2, respectively) (Table 1). In addition, range-weighted richness (Rr) was used to study the carrying capacity of the environment based on the number of PCR-DGGE bands and the percentage of denaturing gradient in the DGGE gel needed to describe the total diversity of the sample analyzed. Rr was statistically higher in 56 and 87 DAH larvae compared to their control groups, indicating higher probability in these intestinal environments to host higher genetic variability (Table 1).

The intensity of a band provides a rough estimate of the proportion of the sequence in a sample, but this may not correlate with cellular relative abundance within the intestine. In order to identify the species corresponding to predominant bands in the DGGE gels, clone libraries of 16S rDNA sequences were prepared from PCR products loaded in DGGE gels. A total of 100 clones per treatment and batch were obtained and sequenced. Mobility of 16S rDNA cloned fragments was compared to the mobility in the original profiles by DGGE, and DNA sequences of clones corresponding to dominant bands or unique

clones determined. DNA sequences of clones were identified at species level (>95% similarity to nearest type strain) and at genus level (at least 90% similarity to nearest type strain) (Table 2).

The total number of operational units (OTUs) was determined based on the number of recombinants obtained with PCR products from pooled intestinal samples for each treatment and fish age. The most frequently OTUs detected in the intestinal microbiota of *S. senegalensis* larvae along the stages studied were identified as γ-Proteobacteria, (percentages ranging from 55% to 68% total clones) regardless the diet administered (Fig. 3). OTUs belonging to the phyla Firmicutes (12%-22% total clones), Actinobacteria (1%- 13% total clones), Mollicutes (1-9% total clones), and unidentified bacteria (5% to 21% total clones) were also detected (Fig. 3).

Sequencing of clones obtained from control diet larvae showed a core of bacteria maintained along the stages sampled. These bands corresponded to OTUs identified as *Enterobacter, Klebsiella, Halomonas, Acinetobacter, Pseudomonas, Alliivibrio, Vibrio, Rhodococcus, Mycoplasma, Anoxybacillus, Geobacillus* genus and *Brevibacillus parabrevis, Vibrio campbelli* and *Photobacterium damselae* subsp *piscicida* species (Fig. 4). Closest relatives as determined by comparative 16S rDNA sequence analysis are summarized in Table 2.

Intestinal microbiota of larvae fed control and probiotic diets shared a total of 10 OTUs comprised by *Pseudomonas, Enterobacter, Halomonas, Acinetobacter* and *Brevibacillus* genera and *Vibrio campbelli* (Fig. 4). Nevertheless a core of 6 OTUs was uniquely present in *S. putrefaciens* Pdp11 larvae (Fig. 4). These OTUs were identified as *Lactobacillus helveticus Pseudomonas acephalitica, V. parahaemolyticus* and *Shewanella* genus. Increased presence of *Vibrio* species was detected in larvae receiving the probiotics. It is worth highlighting that OTUs corresponding to *P. damselae* subsp *piscicida* were not detected in *S. putrefaciens* Pdp11 fed fish, whereas *Shewanella* genus and *Lactobacillus helveticus* were present, representing 10% and 5% of the clones. Conversely, no OTUs were identified as *Shewanella* in microbiota from control larvae, whilst *Photobacterium* comprised between 8% and 1% of the clones (23 to 119 DAH).

In order to break down the contribution of each bacterial group to the observed dissimilarity between *S. putrefaciens* Pdp11 and control samples, similarity percentage analysis (SIMPER) was carried out. In this way, most important species in creating the observed pattern of dissimilarity attributed to probiotic diet at each age were identified. Bacterial groups and contribution to dissimilarity between treated and control groups resulting from SIMPER analysis is summarized in Table 3. Differences in bacterial communities at 56 DAH (start weaning) were attributed to *Shewanella* (17.24%), unidentified bacteria (17.24%), *Acinetobacter* (10.34%), *Rhodococcus* (8.62%) and *Lactobacillus* (8.62%), jointly contributing to 62.06% total dissimilarity compared to control fed fish. In the case of 87 DAH sole differences were attributed to *Vibrio* (18.18%), *Shewanella* (13.64%), *Candidatus* Arthromitus (13.64%) and *Acinetobacter* (12.12%). *P. damselae* subsp. *piscicida* contributed to 11.11% dissimilarity in 23 DAH control larvae. Presence of *P, damselae* subsp. *piscicida* was also detected in samples from control larvae at subsequent developmental stages. At the same time, more than 20% differences were due to *S.*

putrefaciens Pdp11 presence in probiotic fed larvae. This data may be consequence of antagonistic effects exerted by the probiotics on *Photobacterium* cells.

Discussion

The ~~stabilization of the intestinal microbiota is a beneficial characteristic for~~introduction of a probiotic to the larval and fry gut ~~in order to~~may help to avoid the presence of opportunistic and pathogenic bacteria (Hansen and Olafsen 1999: Yang et al. 2012), as well as to develop enhanced immune response (Picchieti et al. 2007; Sun et al. 2013). Dendrograms representing the similarity of the microbial profiles from PCR-DGGE fingerprints clustered probiotic and control samples from 56 and 87 DAH larvae into significantly different groups. In the case of 23 DAH (just metamorphosed) larval fingerprints, although most of probiotic samples clustered together, intra-group similarity was lower. Transition from live to inert feeding is carried out in Senegalese sole from 56 to 87 DAH. This weaning period is critical and frequently yields reductions in sole post-larvae growth rates (Engrola et al. 2009; Mai et al. 2009). Establishment of adequate intestinal microbiota may have important effects on immune tolerance and successful fish rearing (Sugita et al. 1994). In the present study, modifications in sole intestinal microbiota started to be observed at 23DAH, although they were not significant until 56DAH. A transient time seems to be necessary for the establishment of microbial communities characteristic of probiotic treated fish. Similar results were reported by De Schryver et al. (2010) in seabass (*Dicentrarchus labrax*) fed with poly-β-hydroxibutyrate (PHB) supplemented diets.

In the present study, transition from live to inert diet was carried out from 56 to 87 DAH. It is well known that weaning is a critical period in sole rearing and frequently yields reductions in fish growth rates (Engrola et al. 2009; Mai et al. 2009). Growth performance of *S.putrefaciens* Pdp11 fed specimens was significantly enhanced versus Control along weaning (Lobo et al., 2014). Nevertheless, extended growth periods without *S. putrefaciens* Pdp11 supplementation (119 DAH samples), resulted in lower intra-group similarity percentages, indicating that effects of probiotic feeding on larval intestinal microbiota lasted for two months but not reached juvenile stage. A period of 60 day feeding has been reported for *S. putrefaciens* Pdp11 microbiota modulation in sole juveniles (Tapia-Paniagua et al.2010).

Although the DGGE technique may present biases in the detection of bacterial taxa (Woodcock et al. 2007), changes in band abundances as reflected by bacterial diversity can indicate ecological shifts in the characteristics of bacterial communities (Van der Gast et al. 2006; Woodcock et al. 2007). In this context, Shannon index (H') values showed that bacterial evenness for the dominant taxa detected by DGGE in the intestines of *S. putrefaciens* Pdp11 larvae at 56 and 87 DAH were statistically lower compared to those obtained for Control larvae. On the contrary, Rr values were higher in probiotic larvae, reflecting increasing genetic variability of the intestinal microbiota (De Schryver et al. 2010). Differences observed in H' and Rr indicate a shift in the intestinal microbiota of *S. putrefaciens* Pdp11 larvae that can be attributed to the modulation exerted by the probiotic strain, as it has been described in sole juveniles by García de la Banda et al. (2010) and Tapia-Paniagua et al. (2010). In this sense other microorganisms

such as *Bacillus licheniformis* (Kumar et al. 2013), *Pedioccocus acidilactici* (Ferguson et al. 2010) and *Enterococcus faecium* (Avella et al. 2011) proposed as probiotics in aquaculture have been reported as effective digestive microbiota modulators in prawn *Macrobrachium rosenbergii*, turbot (*Scophthalmus maximus*) and Dover sole (*Solea solea*) respectively.

In this study, the majority of bacterial taxa associated with Senegalese sole larvae and fry were identified as γ-Proteobacteria followed by Firmicutes. These bacterial groups are frequently reported as predominant ones in farmed fish such as rainbow trout (*Oncohynchus mykiss*) (Navarrete et al. 2010) and yellow seahorse (*Hippocampus kuda*) (Tanub et al. 2012). On the other hand, γ-Proteobacteria, but not Firmicutes, has also been detected as a predominant group in the intestinal microbiota of weaned *S. senegalensis* specimens (Martin-Antonio et al. 2007; Tapia-Paniagua et al. 2010).

Likewise a core of microorganisms including *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Halomonas*, *Vibrio*, *Alliivibrio*, *Rhodococcus*, *Geobacillus* and *Mycoplasma* genera, and *Brevibacillus parabrevis* and *Photobacterium damselae* subsp *piscicida* were detected in Control larvae along the study. The majority of these microorganisms has not been described in the intestinal microbiota of sole juveniles (Martin Antonio et al. 2007; Tapia-Paniagua et al. 2010). Intestinal fish microbiota is highly influenced by the aquatic environment. In the case of larvae, Ingerslev et al. (2014) reported increasing microbial abundance and diversity after first-feeding and dominance of phylum γ-Proteobacteria in juvenile rainbow trout fed a diet containing fish meal and oil from marine sources. Hoj et al. (2009) found *Artemia* nauplii to be an important source of bacteria that can be transferred to larvae when nauplii are used as live feed. In this context, γ-Proteobacteria have also been reported as the most abundant group in nearly hatched *Artemia* nauplii and *Halomonas*, *Vibrio* and *Pseudomonas* being some of the predominant genera (HØj et al. 2009; Tkavc et al. 2011).

A core of 6 OTUs consisting in *Pseudomonas acephalitica*, *Vibrio parahaemolyticus*, *Lactobacillus helveticus* and *Shewanella* genus, including *S. putrefaciens* Pdp11, was detected in sole specimens. Thus, this core may be considered as a result of the microbiota modulation exerted by *S. putrefaciens* Pdp11. Probiotic modulation was specially marked at 23 DAH when the percentage of probiotic clones was the highest. In this context clones related to *Mycoplasma*, *Anoxybacillus*, *Alliivibrio* and *Rhodococcus* genera, *Candidatus* Arthromitus and *Photobacterium damselae* subsp *piscicida* were not detected ~~like~~as ~~a~~ predominant taxa in the intestinal microbiota of 23 DAH *S. putrefaciens* Pdp11 larvae, whereas they were ~~a dominant taxa present~~ detected in the intestinal microbiota of Control larvae, it thus being dominant taxa. The last two microorganisms have also been detected in the intestinal microbiota of other farmed fish (Cecchini et al. 2012; de Paula Silva et al. 2011). Whereas *P. damselae* subsp *piscicida* is an important pathogen for *S. senegalensis* (Arijo et al. 2005; Zarza and Padrós, 2008), *Candidatus* Arthromitus has been suggested as the aetiological agent of gastroenteritis syndrome in rainbow trout (Urdaci et al. 2001).

It is worthy to highlight the absence or lower presence of *P. damselae* subsp *piscicida* in the intestinal microbiota of *S. putrefaciens* Pdp11 larvae in parallel with increased presence of the probiotic strain. According to Magnadottir (2010), live probiotic bacteria can affect the intestinal microbiota by

their antagonistic activity and/or competition for nutrients and space, reducing the number of pathogenic bacteria. In the present study, *P. damselae* subsp. *piscicida* absence could be related to *S. putrefaciens* Pdp11 ability to inhibit growth and adhesion of this pathogen to the sole intestinal mucus, ~~adhesion of this pathogen~~ as ~~(~~Chabrillon et al. 2005a~~)~~ demonstrated in *in vitro* studies. Although other components cannot be ruled out, adhesion interference could explain the higher resistance against this pathogen reported in sole juveniles fed a diet supplemented with *S. putrefaciens* Pdp11 (Díaz-Rosales et al. 2009; García de la Banda et al. 2010; 2012).

On the other hand, high proteolytic activity levels have been reported in *Lactobacillus helveticus* (Beganovic et al. 2013), as well as different probiotic abilities such as immunomodulation by bioactive peptides released during fermentation (Taverniti et al. 2013; Vinderola et al. 2007), enhancement of goblet cell/colonocyte ratio, the recovery of mucosal atrophy induced by malnutrition (Dock-Nascimento et al. 2007), and antibacterial effect against pathogens mediated by its surface-layer protein and by competitive exclusion (Ahire et al. 2013; Beganovic et al. 2011). *Shewanella* genus has been reported as the predominant bacterial taxa in *S. senegalensis* specimens fed natural prey (Martín-Antonio et al. 2007) and commercial diet (Tapia-Paniagua et al. 2010). *Shewanella* has also been detected in the intestinal microbiota of other fish species such as rainbow trout (Navarrete et al. 2010), goldfish (*Carassius auratus*) (de Paula Silva et al. 2011) and halibut (*Hippoglossus hipoglossus* L.) (Bjornsdottir et al. 2009). In the present study *S. putrefaciens* Pdp11 was cloned and sequenced from 23 to 119 DAH samples as predominant clone from probiotic larvae and fry, thus supporting the ability to colonize reported in previous studies (Lobo et al. 2014; Tapia-Paniagua et al. 2011). The administration of *S. putrefaciens* Pdp11 also resulted in increased numbers of clones corresponding to *Vibrio* genus, especially those identified as *V. parahaemolyticus*. *Vibrio* species have frequently been isolated from seawater and the surface of healthy fish in aquaculture systems (Montes et al. 2006; Chakraborty et al. 2009). Although virulent strains of *Vibrio* can produce several diseases in aquaculture organisms such as prawn (Khuntia et al. 2008), turbot *Scophthalmus maximus* (Villamil et al. 2003b) and Senegalese sole (Zorrilla et al. 2003), their presence in intestinal microbiota of *S. senegalensis* larvae should not always be considered as health risk. In this way, survival rates of control and *S. putrefaciens* Pdp11 groups observed in this study were similar at 119 DAH (88.05-89.83%) (Lobo et al., 2014 comm pers).

In short, this study demonstrates the colonization capability of *S. putrefaciens* Pdp11 and its ability to modulate the predominant bacterial groups of the intestinal microbiota of Senegalese sole larvae. A short pulse (10-30 DAH) of *S. putrefaciens* Pdp11 feeding results in increased levels of *Vibrio* genus and decreased the presence of the fish pathogen *Photobacterium damselae* subsp *piscicida*. This modulation may constitute an effective tool in a suitable *S. senegalensis* larviculture performance.

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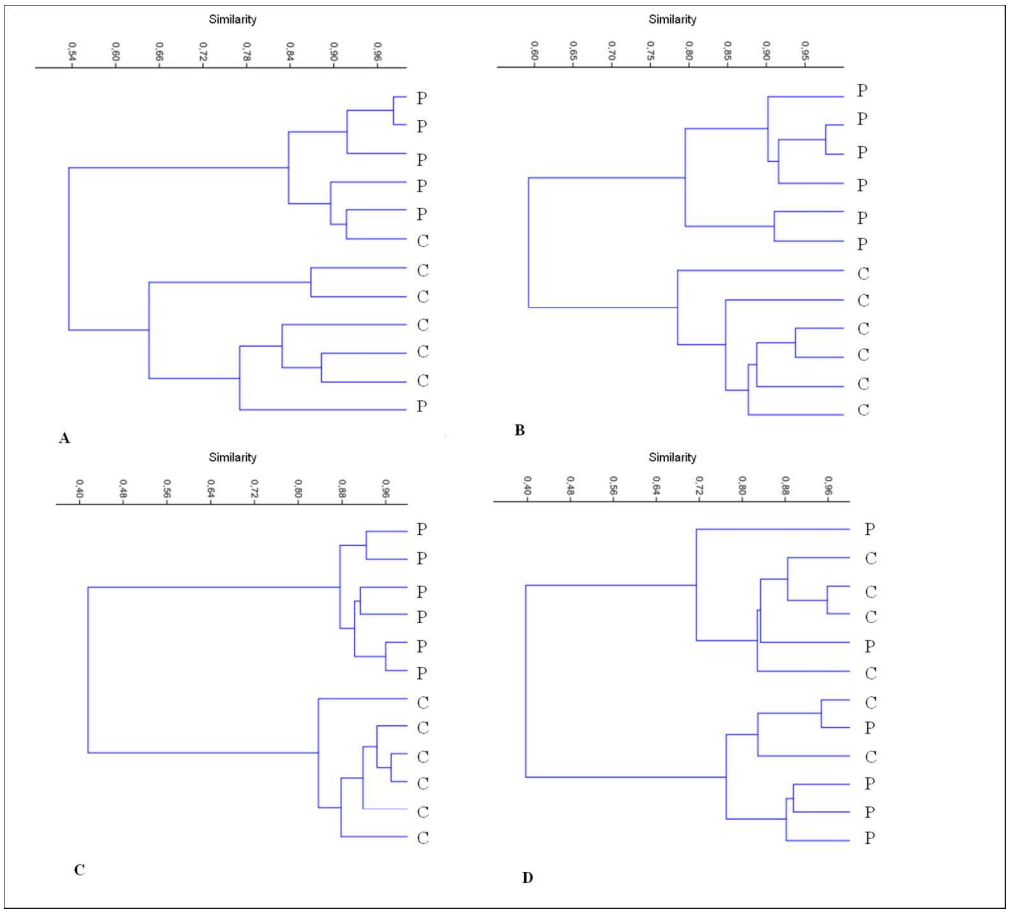

Figure 1. Cluster analysis of 16S rDNA PCR-DGGE profiles of individual sole specimens fed control (C) and probiotic (Shewanella putrefaciens Pdp11) supplemented (P) diets from 10 to 30 days after hatching (DAH). Samples were obtained from larval intestines at 23 DAH (A), 56 DAH (B), 87 DAH (C) and 119 DAH (D). Cluster analysis was performed using UPGMA methods based on Bray-Curtis similarity index obtained from densitometric curves.
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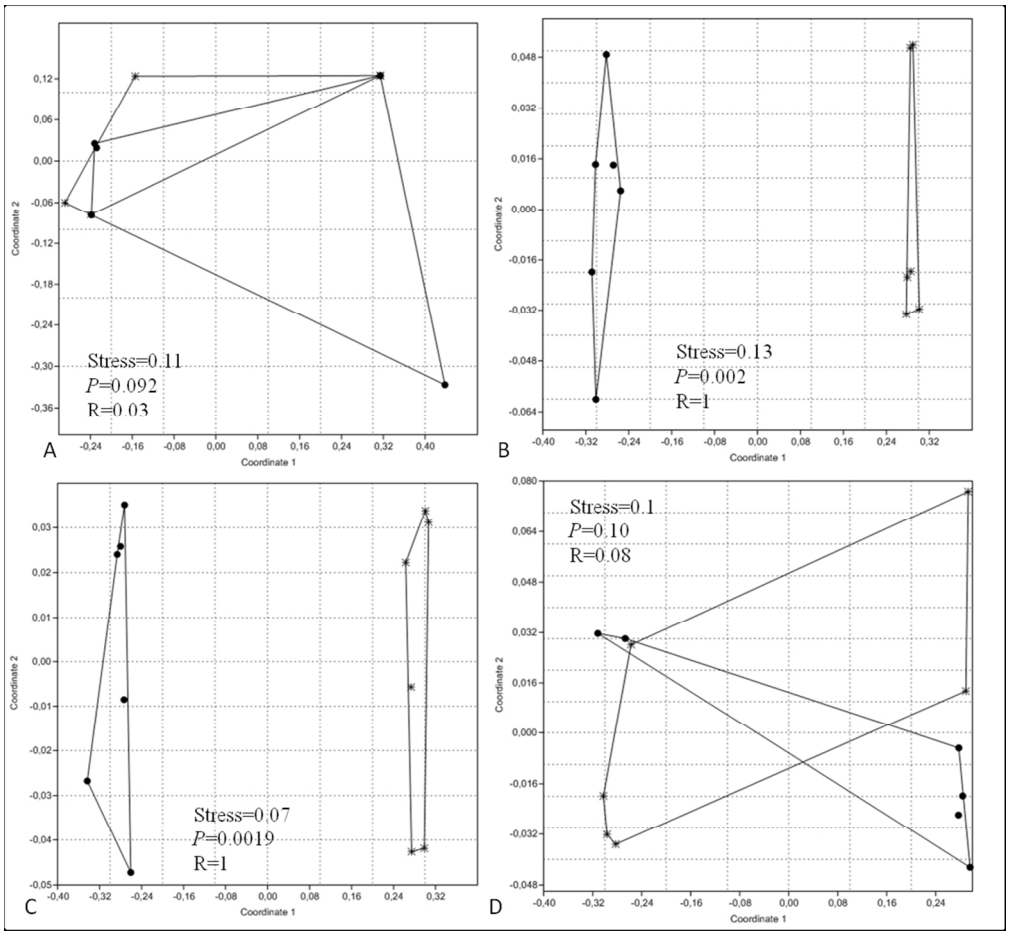

Figure 2. Non-metric multidimensional scaling (NMDS) plots using Bray-Curtis index of PCR-DGGE profiles associated to the intestines of sole specimens fed control (circles) and probiotic (Shewanella putrefaciens Pdp11) supplemented (asterisks) diets from 10 to 30 days after hatching (DAH). Samples were obtained from intestines at 23 DAH (A), 56 DAH (B), 87 DAH (C) and 119 DAH (D).
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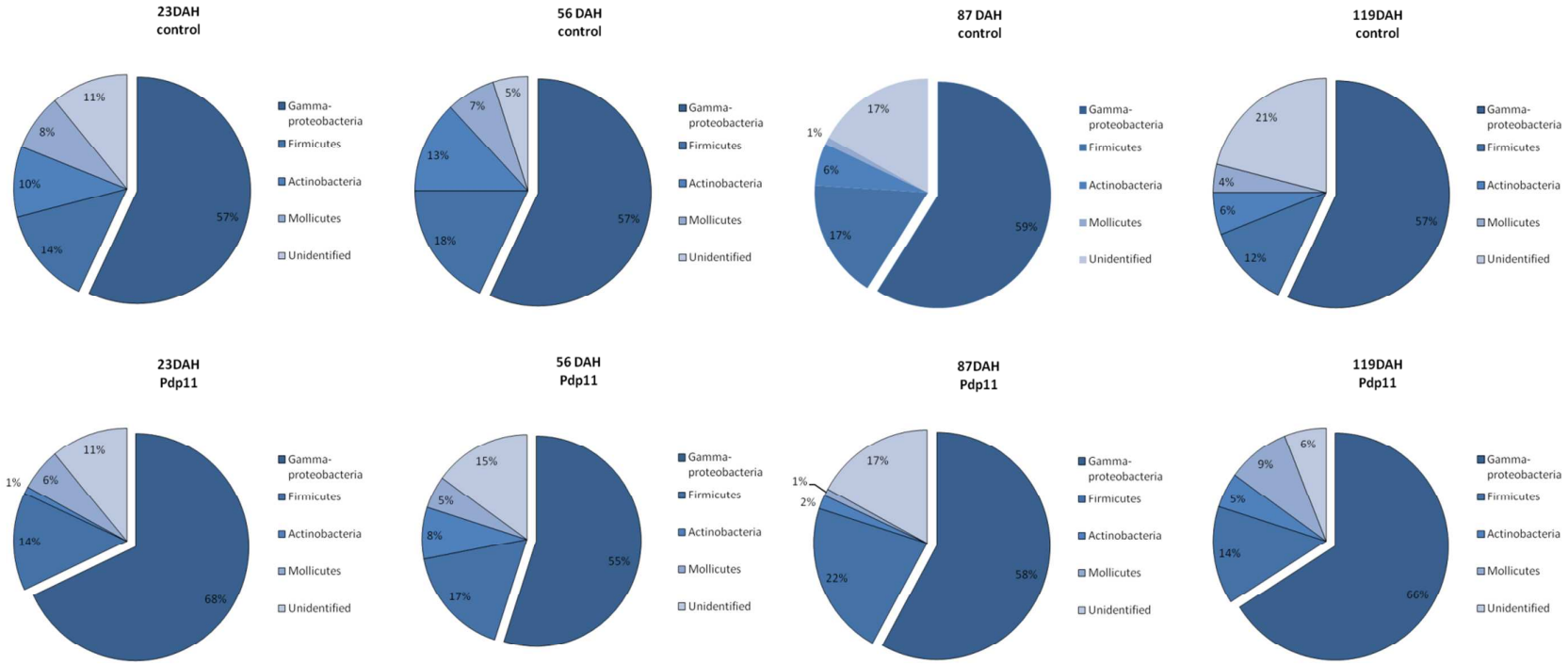

Figure 3. Relative abundance of bacterial groups detected in the intestines of sole specimens fed control (control) and *S. putrefaciens* Pdp11 supplemented (Pdp11) diets from 10 to 30 days after hatching (DAH). Samples were obtained from intestines at 23 DAH (A), 56 DAH (B), 87 DAH (C) and 119 DAH (D). Percentages were calculated relative to the total number of clones obtained for each treatment and fish developmental stage.

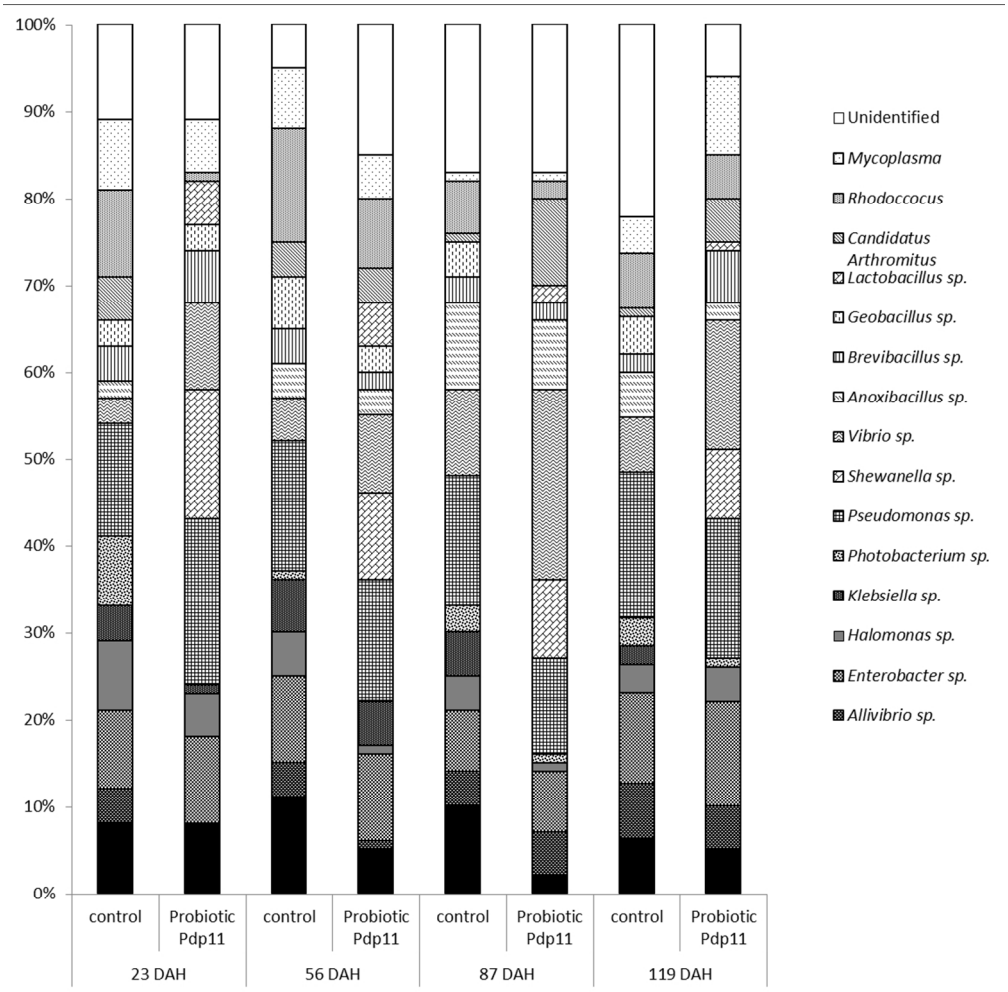

Figure 4. Relative abundance of bacterial groups detected in the intestines of sole specimens fed control and probiotic (Shewanella putrefaciens Pdp11) supplemented diets from 10 to 30 days after hatching (DAH). Samples were obtained from intestines at 23 DAH, 56 DAH, 87 DAH and 119 DAH. Percentages were calculated relative to the total number of clones obtained for each treatment and fish developmental stage.
194x192mm (150 x 150 DPI)

Table 1. Shannon diversity index (H'), species richness (R) and range-weighted richness (Rr) values of intestinal microbiota DGGE patterns of Senegalese sole larvae fed Control and Probiotic (*Shewanella putrefaciens* Pdp11) diets and sampled at 23, 56, 87 and 119 days after hatching (DAH). Fish were fed probiotic diet from 10 to 30 DAH. Data expressed as mean ± standard deviation.

DAH	Diet	H'	R	Rr
23	Control	2.6 ± 0.2	23.0 ± 5.0	105 ± 20.50
	Probiotic	2.3 ± 0.2	24.0 ± 3.0	115 ±10.75
56	Control	2.6 ± 0.1	31.0 ± 2.0	115 ± 9.23
	Probiotic	2.1 ± 0.2*	31.0 ± 2.0	192 ± 10.11*
87	Control	2.6 ± 0.2	25.0 ± 3.0	125 ± 15.25
	Probiotic	2.1 ± 0.1*	31.3 ± 3.0	192 ±12.20*
119	Control	2.8 ± 0.1	25.3 ± 2.0	125 ± 10.15
	Probiotic	2.5 ± 0.1	26.0 ± 2.0	135 ± 9.70

Significant differences compared to the control group are noted by asterisk.

Table 2. Nearest-match identification of 16S rDNA sequences of clone libraries from intestines of soles fed Control (C) and S. putrefaciens Pdp11 supplemented (P) diets from 10 to 30 days after hatching (DAH). Percentages are relative to the total number of clones obtained for each treatment and fish developmental stage. Fish were sampled at 23, 56, 87 and 119 DAH.

Closest relative	Similarity (%)	GenBank accession	Clone percentage							
			23 DAH		56 DAH		87 DAH		119 DHA	
			C	P	C	P	C	P	C	p
γ-Proteobacteria										
Acinetobacter sp MBL 11-0202	98	HM366450	4	5	5	1	5	1	4	0
Acinetobacter sp CmNA3	99	HM352317	4	3	6	4	5	1	2	5
Allivibrio sp SW5-1	98	FR744854	4	0	4	1	4	5	6	5
Enterobacter sp 11H	97	HM803943	8	6	5	4	6	6	5	9
Enterobacter sp CC-SN15-5	99	EU596390	1	4	5	6	1	1	5	3
Halomonas sp AMP12	97	HM104378	8	5	5	1	4	1	8	4
Klebsiella sp PG4-2	97	AB277851	4	1	6	5	5	0	2	0
Photobacterium damselae subsp *piscicida* pRDA19	98	AJ749800	8	0	1	0	3	1	3	1
Pseudomonas	99	IQ766121	8	7	2	4	3	3	6	8
Pseudomonas sp FG-12b	99	JF724071	4	4	4	3	5	0	2	0

Pseudomonas sp Iso-13	95	KC768739	0	2	4	0	4	5	3	2
Pseudomonas sp Ant-4	98	HF678977	1	0	5	4	1	0	2	0
Pseudomonas acephalitica Ind01	99	AM407893	0	4	0	1	0	3	0	6
Pseudomonas fluorescens LMG 5329	95	JQ974027	0	2	0	2	3	0	4	0
Shewanella putrefaciens pdp11	98	JX415533	0	7	0	6	0	4	0	3
Shewanella ANA	99	CP00046	0	8	0	4	0	5	0	5
Vibrio sp DMS01	97	FJ463236	0	0	4	1	5	5	0	0
Vibrio campbelli HNS034	99	JN128268	2	2	1	1	5	6	6	6
Vibrio natriegens 01/252	98	AJ874353	1	0	0	0	0	5	0	5
Vibrio parahaemolyticus qdfsVp001	98	JN108879	0	2	0	1	0	1	0	0
Vibrio sp Pelg0901	98	JF330909	0	6	0	6	0	5	0	4
Actinobacteria										
Rhodococcus erythropolis H11	99	EF204435	8	0	6	6	2	2	5	5
Rhodococcus sp TM1	98	AY642534	2	1	7	2	4	0	1	0
Firmicutes										
Anoxybacillus sp D503	97	FJ430046	0	0	2	1	6	3	0	0
Uncultured *Anoxybacillus* sp K.b-8	97	HM031453	2	0	2	2	4	5	5	2
Brevibacillus paravebris DYJK58	98	JX415533	4	6	4	2	3	2	2	6

Candidatus Arthromitus sp SFB-rat-Yit	99	NR074540	0	0	2	2	0	4	0	2
Candidatus Arthromitus sp SFB-rat-Yit	99	NR074545	5	0	2	2	0	4	0	2
Candidatus Arthromitus	98	D86303	0	0	0	0	0	2	0	1
Geobacillus sp V1W70BlkII	98	FN556447	3	3	6	3	4	0	4	0
Lactobacillus helveticus GIMC23:KSN	98	JF728275	0	5	0	5	0	2	0	1
Mollicutes										
Mycoplasma microti IL371	98	FJ609188	4	6	4	2	0	0	0	2
Uncultured *Mycoplasma* sp Kb.1	99	HM031446	4	0	3	3	1	1	2	9
Unidentified microorganisms										
Marine bacterium B37	96	AB607159	8	7	2	4	3	3	6	8
Bacterium A119	98	HQ332151	3	4	1	5	4	1	4	2
Bacterium PJ-35	95	KF146331	0	1	0	2	3	5	6	0
Uncultured bacterium R4J7L4_B3	95	GQ467872	0	0	0	2	3	0	4	0
Uncultured bacterium ncd89b06c1	98	HM256348	0	0	1	4	3	3	3	1
Uncultured bacterium BR01AD06	98	DQ857141	0	0	0	0	0	4	0	1

Table 3. SIMPER analysis performed to identify contribution of bacterial groups to total dissimilarity between control and probiotic fed *Solea senegalensis* larvae. Values correspond to dissimilarity percentages between probiotic and control groups. A total of 100 cloned sequences were identified for each treatment and fish developmental stage.

Group	Dissimilarity percentage (%)			
	23 DAH	**56DAH**	**89DAH**	**119DAH**
Acinetobacter	0	**10,34**	**12,12**	1,58
Alliivibrio	5,55	5,17	1,51	1,58
Enterobacter	1,38	0	0	3,17
Halomonas	4,16	**6,89**	4,54	1,58
Klebsiella	4,16	1,72	7,57	3,17
Photobacterium damselae subsp. *piscicida*	**11,11**	1,72	3,03	3,17
Pseudomonas	8,33	1,72	6,06	0
Shewanella	**20,83**	**17,24**	**13,64**	12,7
Vibrio	**9,77**	**6,89**	**18,18**	**14,29**
Anoxibacillus	2,77	1,72	3,03	4,76
Brevibacillus	2,77	3,44	1,51	6,34
Geobacillus	0	5,17	6,06	6,34
Lactobacillus	6,94	8,62	3,03	1,58
Candidatus Arthromitus	6,94	0	**13,64**	6,34
Rhodococcus	**12,5**	**8,62**	6,06	1,58
Mycoplasma	2,77	3,44	0	**7,93**
Unidentified	0	**17,24**	0	**23,81**

Values in bold represent percentages contributing to 50% dissimilarity in each group.